

## Development of Resistance to *Raspberry Bushy Dwarf Virus* in ‘Meeker’ Red Raspberry

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### Abstract

*Raspberry bushy dwarf virus* (RBDV) has been a reemerging problem over the past 15 years throughout raspberry growing regions of the world. This is due primarily to growers planting susceptible cultivars that have superior fruit quality. Crumbly fruit and yield reduction can combine to reduce crop value by more than 50%. RBDV is pollen-borne, making chemical control virtually impossible. ‘Meeker’ raspberry plants were transformed with the coat protein gene, mutated forms of the movement protein gene or nontranslatable RNA of RBDV. The presence of these viral sequences was confirmed by DNA hybridization analysis and RT-PCR of DNase digested total RNA preparations. Of 197 transgenic lines planted in replicated blocks in the field under extreme disease pressure, five lines remained free of RBDV after four years. All 202 of the wild-type ‘Meeker’ plants in the same plot were infected with RBDV at the end of four years. Most transgenic lines had 9 of 9 plants infected and a few lines showed partial resistance to infection, with 1 to 6 of the 9 plants infected. The five lines showing field resistance were also resistant to RBDV when tested by grafting. Fruit evaluations of these five lines showed that they contained the five anthocyanins at the same relative concentrations as were present in wild type ‘Meeker’ fruit. Field and fruit evaluations of these five lines will be continued for another two years.

### INTRODUCTION

Raspberry production in the Pacific Northwest, Oregon and Washington in the USA and southwestern British Columbia in Canada, has increased dramatically during the 1990s. The increase in production has been the result of a move to more mechanical harvesting of raspberries that resulted in reduced labor costs, a change in cultivars and an increase in acreage. Prior to 1980, ‘Willamette’ was the most widely planted raspberry cultivar in this region. Since the early 1980’s, ‘Meeker’ has become the cultivar of choice for most growers because it outyields ‘Willamette’, has an intermediate level of resistance to root rot and produces a higher quality berry for the valuable fresh and whole frozen berry markets. Other cultivars have replaced ‘Meeker’ in the fresh market but it still makes up over 70% of the processed market including the valuable whole frozen berry market.

With the change in cultivars and increased planting density there was a rapid increase in the incidence of *raspberry bushy dwarf virus* (RBDV), since ‘Meeker’ is susceptible to the virus while ‘Willamette’ is immune (Daubeny et al., 1982). The resistance breaking strain of RBDV that infects ‘Willamette’ and other cultivars with the *Bu* gene, and found in Europe (Barbara et al., 1984; Knight et al., 2002) has not been identified in North America. In surveys in the 1990’s it was found that RBDV spreads rapidly in fields in Washington and British Columbia and more slowly in the Willamette Valley in Oregon and southern Washington (Martin, 1998). Also, at that time RBDV was found in ‘Marion’ blackberry in the Willamette Valley (Strik and Martin, 2003). ‘Marion’ has been planted in the Willamette Valley since the mid-1960’s but this was the first report of RBDV in this cultivar in North America. It can spread quite rapidly through a ‘Marion’ field as well as in red raspberry. In ‘Marion’ it causes a severe druplet abortion which leads to distorted fruit and a yield loss of about 40%.

'Meeker' red raspberry has been developed that contain several different RBDV constructs including: nontranslatable RNA, coat protein, and three different mutated movement protein constructs (Martin et al., 2001). This paper describes the initial greenhouse and field evaluations of the transgenic plants in terms of virus resistance and fruit quality.

## **MATERIALS AND METHODS**

Transgenic lines of 'Meeker' were grafted twice in the greenhouse with RBDV and planted in field plots in Washington and Oregon along with wild-type 'Meeker' plants. Standard commercial management practices were applied in the plantings including; the use of raised beds to minimize the risk of root rot, pruning, fertilizer applications, spray schedules and trellising. Grafted plants were maintained in a screenhouse and tested for RBDV by ELISA after 60 days and again after going through a dormant season. The field trial plants were tested for the presence of RBDV by ELISA annually (Martin, 1998). Fruit was harvested by hand and visual appearance judged by a panel that was unfamiliar with the planting or the constructs used. For fruit analysis, hand harvested fruit was frozen in liquid nitrogen immediately after harvest in the field and transported back to the laboratory in liquid nitrogen. Fruit was maintained at -80°C until the anthocyanin analysis was completed. Anthocyanin content was determined using standard methods.

### **Virus Testing and Gene Analysis**

Three leaflets per plant were collected from the transgenic field trials in Oregon and Washington. Each sample was tested in duplicate for RBDV. Known infected raspberry plants maintained in a greenhouse were used as positive controls in each of the tests. Southern analysis using single- and double-digested DNA was carried out on a subset of plants to confirm that the inserted genes were present. Total nucleic acid was extracted from 28 transgenic lines and three healthy controls, with at least five representative lines containing each construct, digested with RQ1 RNAase free DNAase and the resulting nucleic acid used in RT-PCR tests. The upstream primer was from the sequence in the promoter region and the downstream primers were approximately 500 bases downstream in either the CP or MP. The same downstream primer was used for all MP constructs and the nontranslatable RNA. All extractions, enzymatic reactions and Southern analysis were performed using standard methods (Sambrook et al., 1989).

## **RESULTS**

In the RT-PCR test the presence of transcripts was detected in 28/28 transgenic lines analyzed but not in wild type 'Meeker'. At least five lines from each construct along with three healthy controls were analyzed in these tests. When the same nucleic acid preparations were used in PCR reactions without an RT step, there was no product detected, suggesting that the RQ1 DNase digestions were effective and the amplicons obtained were dependent on the presence of transcripts rather than the inserted DNA.

Approximately 40 different transgenic lines were planted out for each of the five constructs used in the study (Table 1). Many lines produced crumbly fruit even though they were not infected with RBDV. The percentage of lines that produced crumbly fruit did not vary between constructs, suggesting that crumbly fruit resulted from the transformation process rather than because of the transgenes used. Some plants produced large leaves having almost twice the leaf area of leaves of the nontransgenic plants and often these plants were quite short and produced crumbly fruit. Again, there was no relationship between the large leafed stunted plants and the construct used to develop the plant.

The planting in Washington was under extreme disease pressure since it was surrounded by four to six rows of RBDV infected 'Meeker' plants. This planting had three replicated blocks of the test plants. In each replicate, there was a three plant plot of each of the transgenic lines and there were also 202 wild-type 'Meeker' plants in the

planting. All of the 202 wild-type 'Meeker' plants in the trial were infected with RBDV four years after planting. Four years after the trial was planted in the field only five lines were free of RBDV in each of the three replicate blocks. There were 19 lines that had between one and six of the nine plants infected with RBDV, but most lines had nine of nine plants infected (Table 1). The planting in Oregon was not subjected to disease pressure. The lines that produced crumbly quality fruit in the first two years were the same in Oregon and Washington, suggesting that the crumbly fruit observed in these plants was due to the transformation process since none of the plants in the Oregon trial were infected with RBDV even after four years in the field. The crumbly fruit from the non-infected transgenic lines was similar in appearance to fruit from RBDV infected plants.

The fruit of the 24 lines that did not have 100% infection after four years in the field were evaluated for anthocyanin content. Each of the 24 lines produced the same five anthocyanins, in the same ratios and at the same levels as wild-type 'Meeker' plants (Fig. 1). Anthocyanin content did not differ between the plantings in Oregon and Washington.

## DISCUSSION

The results presented here demonstrate that 'Meeker' has been modified genetically to produce resistant lines with fruit quality that is comparable to wild-type 'Meeker'. Also, for some unknown reason there was a high number of off-type plants that resulted from the transformation process. Each of the constructs used resulted in some off-type plants, suggesting that this affect is not the result of the genes used. One of the constructs made use of nontranslatable RNA which had the start codon removed and three stop codons inserted in the first 60 nucleotides of the sequence. The construct with nontranslatable RNA also gave off-type plants suggesting that the problem is with the transformation process rather than the presence of the RBDV genes. Another construct (2173) gave 100% off-type plants. Currently, 'Meeker' raspberry is being transformed with vector that does not contain any viral sequences to determine the rate of off-type plants in the absence of any RBDV sequences and if the off-types result from the transformation process. Resistant lines were obtained with only two constructs; three were obtained with the nontranslatable RNA and two with one of the movement protein deletions. None of the coat protein lines exhibited resistance to RBDV.

Anthocyanin content was not altered statistically in the transgenic plants analyzed for this trait. Each line produced the same five anthocyanins that were detected in wild-type 'Meeker' plants and in the same ratios. Volatiles or aroma components and machine harvestability will be evaluated in 2004 and 2005 in larger plantings of the five transgenic lines that exhibited resistance to RBDV.

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**Tables**

Table 1. Summary of field tests and evaluations on transgenic raspberry plants with various RBDV constructs.

Construct	Total # Evaluated <sup>a</sup>	Off-Type Lines <sup>b</sup>	Off-Type Fruit <sup>c</sup>	Less than 100 Infected <sup>d</sup>	#Resistant/#Grafted (2X) <sup>e</sup>
2171	48	35	3	10	12/35
2172	29	16	8	5	15/26
2173	36	32	4	0	8/30
2174	49	35	7	7	7/20
2178	29	23	4	2	7/26
Totals	191	141	26	24	49/137

<sup>a</sup>The number of lines with each construct that were included in the field trials.

<sup>b</sup>Number lines that had off-type plants in the field trial.

<sup>c</sup>Number of lines that had off-type fruit on otherwise normal looking plants.

<sup>d</sup>Number of lines for each construct that were less than 100% infected after four years.

<sup>e</sup>Plants that showed crumbly fruit in the field before grafting was completed were not grafted

## Figures

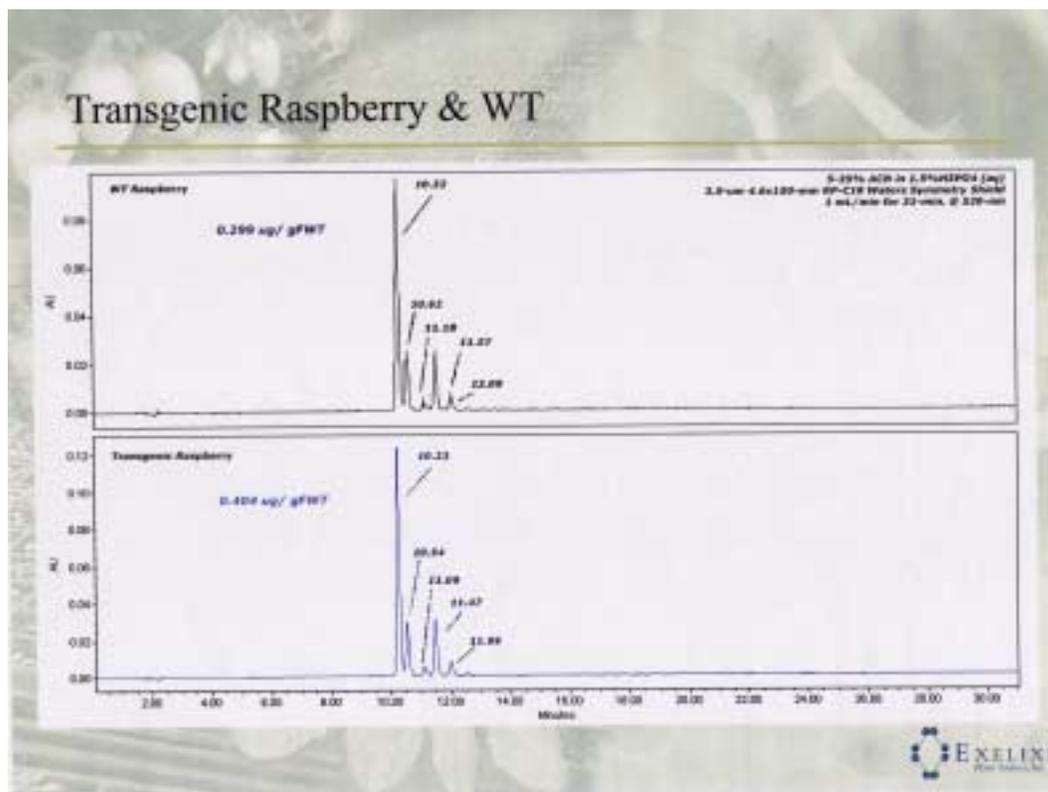


Fig. 1. Representative scan of anthocyanins from wild-type (top) and one transgenic line (bottom) showing that all five anthocyanins were present in both lines and in the same relative concentrations. In this case the transgenic line had slightly higher levels of anthocyanins than the wild-type 'Meeker'.